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A survey of the invertebrates feeding
on living clover roots (*Trifolium repens* L.)
using ^{32}P as a radiotracer

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With 2 figures

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1. Introduction

Although the impact of herbivores on grassland has attracted much attention from ecologists, almost all studies have been devoted to the consumption of above ground herbage. Root feeders have only been considered when their activities cause a dramatic decline in plant vigour or yield. In such cases the numbers of root feeders involved can be several millions per acre, as seen in leatherjacket (WHITE & FRENCH 1968) or wireworm (Anon., 1941) outbreaks. For many years, the ecological significance of root feeding herbivores remained unexamined (COLEMAN & SASSON 1980). Recently some of the I.B.P. grassland studies indicated that the majority of the primary production of grassland is consumed by soil organisms. SCOTT *et al.* 1979 suggested that 7—26 % of the total consumption was of underground parts. However, this estimate relied on a total extraction of invertebrates, assumptions about what they were eating and then calculations of the amount consumed from the biomass of the animals present. Such calculations depend crucially on the validity of the assumptions about what the various components of the soil fauna eat and the aim of this survey was to discover, by direct measurement, which invertebrates were feeding on living roots using clover *Trifolium repens* L. as the model plant.

2. Materials and methods

2.1. The study site

The study area at Henfaes farm, Aber, North Wales (Grid ref. SH654733) had been left as permanent pasture for well over fifty years (PETERS 1980) and was grazed by sheep. The soil was a brown earth with gleying, containing over 13 % clay and with a pH of 6.0—6.3 (BALL 1963). The area was 9.3—11.0 m above sea level, was close to the sea and contained 48 plant species with *Trifolium repens* and *Lolium perenne* dominants (TURKINGTON & HARPER 1979). Because sheep were often held at a high stocking rate, the litter layer was minimal and contained a sparse soil fauna (THISTLETON 1975; BAYLIS 1984).

2.2. Labelling *T. repens* with ^{32}P

Preliminary laboratory experiments were conducted to see if ^{32}P topically applied to leaves would give a satisfactory level of label in the roots, without to any significant extent leaking out into the surrounding soil.

Sixteen white clover seedlings (*T. repens* var. 5100) were grown in compost in the greenhouse, and after twelve weeks the soil was washed from the roots and the plants transferred to glass fronted observation chambers (19 cm × 17 cm × 7 cm). One side of the root system was in contact with the glass face which was covered with black polythene to exclude light. The plants were left until they recommenced growing, when they were labelled with 50 μCi ^{32}P in orthophosphate which was applied to the leaves in droplets using a "Hamilton" syringe. After six hours, the leaves and stolons were severed at the soil surface and autoradiographs were made of the roots *in situ* by removing the

glass covering the roots and replacing it with a thin sheet of clear polythene. This, unlike the glass did not significantly attenuate the radiation and it prevented chemical fogging of the X-ray film by the soil. A sheet of X-ray film (Kodak, Noscreen) was placed against the polythene and was then covered with hardboard and black polythene to prevent light fogging. The film was exposed for four days.

After exposure, the roots were carefully removed from the soil and placed on "Benchcote"¹⁾. Autoradiographs were similarly made of the roots, and of the soil surface from which they had been removed.

The autoradiographs showed that the ³²P was distributed unevenly in the clover roots (Figure 1), accumulating in root tips, root nodules and young tissue. Over the test period, no detectable quantities of isotope leaked from the roots to the surrounding soil. In six hours plants were sufficiently labelled for the roots to be readily detectable. The labelling technique was very simple and easily applied in the field.

2.3. Sampling the root-feeding fauna

A permanent 10 m × 10 m grid was marked out in the study pasture and on each sampling period, six stolons of *T. repens* were located at random within the grid. ³²P solution was placed on the foliage, and to prevent disturbance, the labelled plants were caged with open-ended wire boxes (30 cm × 30 cm × 10 cm). The top of each box was covered with transparent polythene to keep out the rain and the whole was fixed to the ground with steel pegs. As the ³²P was observed to stay on the leaves in the laboratory, no shield was placed between the labelled leaves and the soil, in the field.

After a six hour labelling period, the shoot system of each plant was severed to ground level to stop further translocation to the roots, and placed in numbered polythene bags. The roots, together with surrounding soil, were carefully dug up in 15 cm × 15 cm × 10 cm blocks. These were taken to the laboratory in individual sealed and numbered tins. Finally, the surrounding field was monitored for contamination, with a geiger counter and if any was found, the material was removed.

In the laboratory, a subsample of soil (1 cm³) was taken from each block and ectoparasitic nematodes were extracted for 24 hours in a modified Baermann funnel (BAYLIS 1984).

Endoparasitic nematodes were studied separately. A second core (7.5 cm diameter × 5 cm deep) was removed from each block, and the soil mesofauna were extracted for five days using a modified Macfadyen high gradient extractor (THISTLETON 1975). Finally, using a Tullgren funnel, the soil macrofauna were extracted over one week from the remains of the original block.

After a trial run in April 1980, the macrofauna were handsorted from the remains of the original core before the soil was put in the Tullgren funnel. This took one hour.

Labelled animals were detected by autoradiography and the gut contents of radioactive earthworms were examined microscopically. The nematodes were removed from the Baermann funnel collecting fluid with a pasteur pipette and were placed on "Benchcote". The earthworms removed by hand-sorting were killed in boiling water and the surface of each was washed in distilled water. Each earthworm was dissected to expose the alimentary tract and placed on "Benchcote". In all cases, the "Benchcote" and the fauna were covered with a thin layer of polythene to prevent chemical fogging of the superimposed X-ray film and a sheet of 3 mm glass was placed on top of each layer to prevent cross-contamination when the samples were stacked on top of each other for the 4-day exposure.

The whole sampling procedure was repeated every four months between August 1980 and August 1981. The only modification was in April 1981 when twelve plants were labelled, six for six hours and six for twenty-four hours to see the effects of an extended labelling period.

3. Results

The numbers and types of soil organisms removed from the soil were noted, together with those which were radioactive (Table 1).

Over the year, 4 (14.3%) of the 28 dipteran larvae caught were radioactive 27 being *Bibio marci* L., and one a leatherjacket (Tipulidae). Six (23%) out of 26 coleopteran larvae contained ³²P. Not all could be identified to family but five of the six containing ³²P were weevil larvae, the other being a chafer grub (*Phyllopertha horticola* L.). In general, few beetle larvae were found in the field. A small proportion, 6 (0.9%) of the 672 Collembola caught were radioactive, and these were mainly from the family Entomobryidae. A similar proportion (0.3%) of the ectoparasitic nematodes showed activity, but no mites were radiolabelled.

The autoradiographs showed that the earthworms *Aporrectodea longa* (UDE), *A. caliginosa* (SAVIGNY), *Lumbricus rubellus* (HOFFMEISTER) and a number of enchytraeids had acquired radioactivity (Figure 2, Table 2).

¹⁾ Benchcote is made by Whatman's Ltd.

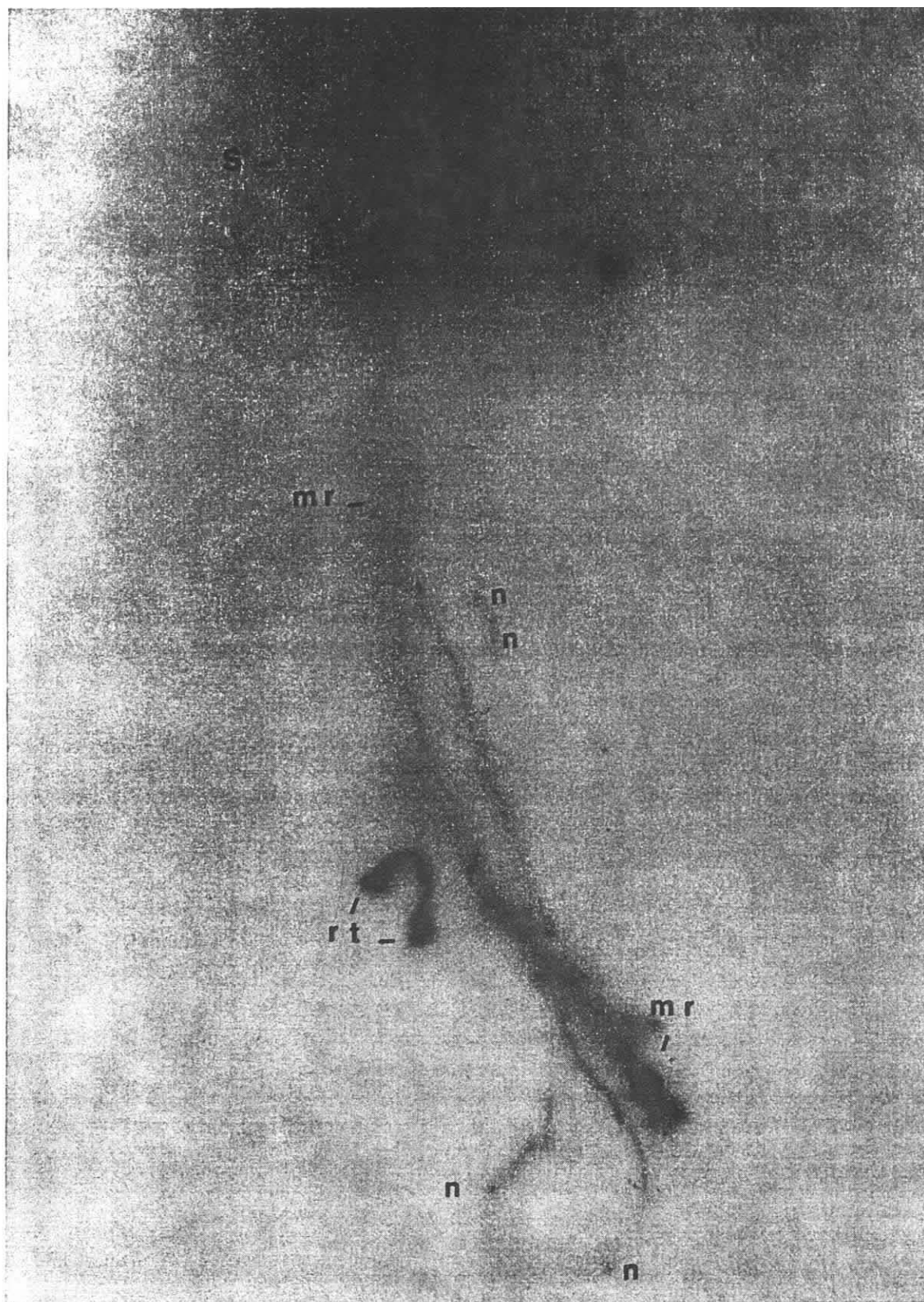


Fig. 1. Autoradiograph of ^{32}P labelled roots of clover (*Trifolium repens*). Plant labelled for 6 h, film exposed for 1 day. mr — main root; n — nodule; rt — root tip; S — stolon.

Microscopic examination of radioactive earthworm gut contents after autoradiography did not reveal any recognisable root fragments.

Although autoradiographs give only a qualitative indication of the amount of radio tracer present, inspection of the intensity and extent of film fogging produced by those groups which took up tracer suggested the following order of importance. Annelids > coleopteran larvae > dipteran larvae > Collembola.

Table 1. The proportions of soil-dwelling animals found to have taken-up ^{32}P and so presumed to have been living on *Trifolium repens* roots during the year long survey of a pasture

	Annelids A	Annelids B	Acarina	Collembola	Nematoda	Diptera (larvae)	Coleoptera (larvae)	Coleoptera (adults)	Araneae
April 1980									
No. caught	—	18	1	10	58	4	4	0	0
No. with ^{32}P	—	4	0	2	0	1	0	—	—
% with ^{32}P	—	22.2	0	20.0	0	25.0	0	—	—
August 1980									
No. caught	30	67	64	192	269	4	1	1	1
No. with ^{32}P	11	8	0	2	0	0	0	0	0
% with ^{32}P	36.7	11.9	0	1.0	0	0	0	0	0
December 1980									
No. caught	43	70	18	53	107	4	9	5	0
No. with ^{32}P	8	0	0	0	2	1	1	0	—
% with ^{32}P	18.6	0	0	0	1.9	25.0	11.1	0	—
April 1981 (6 h)									
No. caught	19	32	18	14	98	8	1	0	1
No. with ^{32}P	4	8	0	0	0	0	1	—	0
% with ^{32}P	21.1	25.0	0	0	0	0	100.0	—	0
April 1981 (24 h)									
No. caught	8	37	13	43	99	5	1	0	1
No. with ^{32}P	1	11	0	2	1	1	1	—	0
% with ^{32}P	12.5	29.7	0	4.6	1.0	20.0	100.0	—	0
August 1981									
No. caught	5	0	83	360	323	3	10	3	0
No. with ^{32}P	0	—	0	0	0	1	3	0	—
% with ^{32}P	0	—	0	0	0	33.3	30.0	0	—
Grand Total									
No. caught	105	224	197	672	863	28	26	9	3
No. with ^{32}P	24	31	0	6	3	4	6	0	0
% with ^{32}P	22.9	13.8	0	0.9	0.3	14.3	23.1	0	0

A hand-sorted earthworms; B heat-extracted annelids (earthworms and enchytraeids). In isolated cases, specimens of the following were found: one slug (December 1980), 41 proturans (April 1980), 149 ants (August 1981), but none was radio-labelled.

Table 2. The species of annelids obtained by hand or by heat extraction and the proportions which had taken up ^{32}P

Species	No. Labelled		No. Unlabelled		% Labelled
	Hand	Heat	Hand	Heat	
<i>Aporrectodea caliginosa</i>	11	9	9	5	58.8
<i>A. rosea</i>	0	0	3	1	0
<i>A. longa</i>	3	2	1	3	55.6
<i>Allolobophora chlorotica</i>	0	0	2	0	0
<i>Lumbricus rubellus</i>	10	10	53	30	19.4
Enchytraeidae	0	10	13	152	5.7
Unidentified	0	0	2	0	0

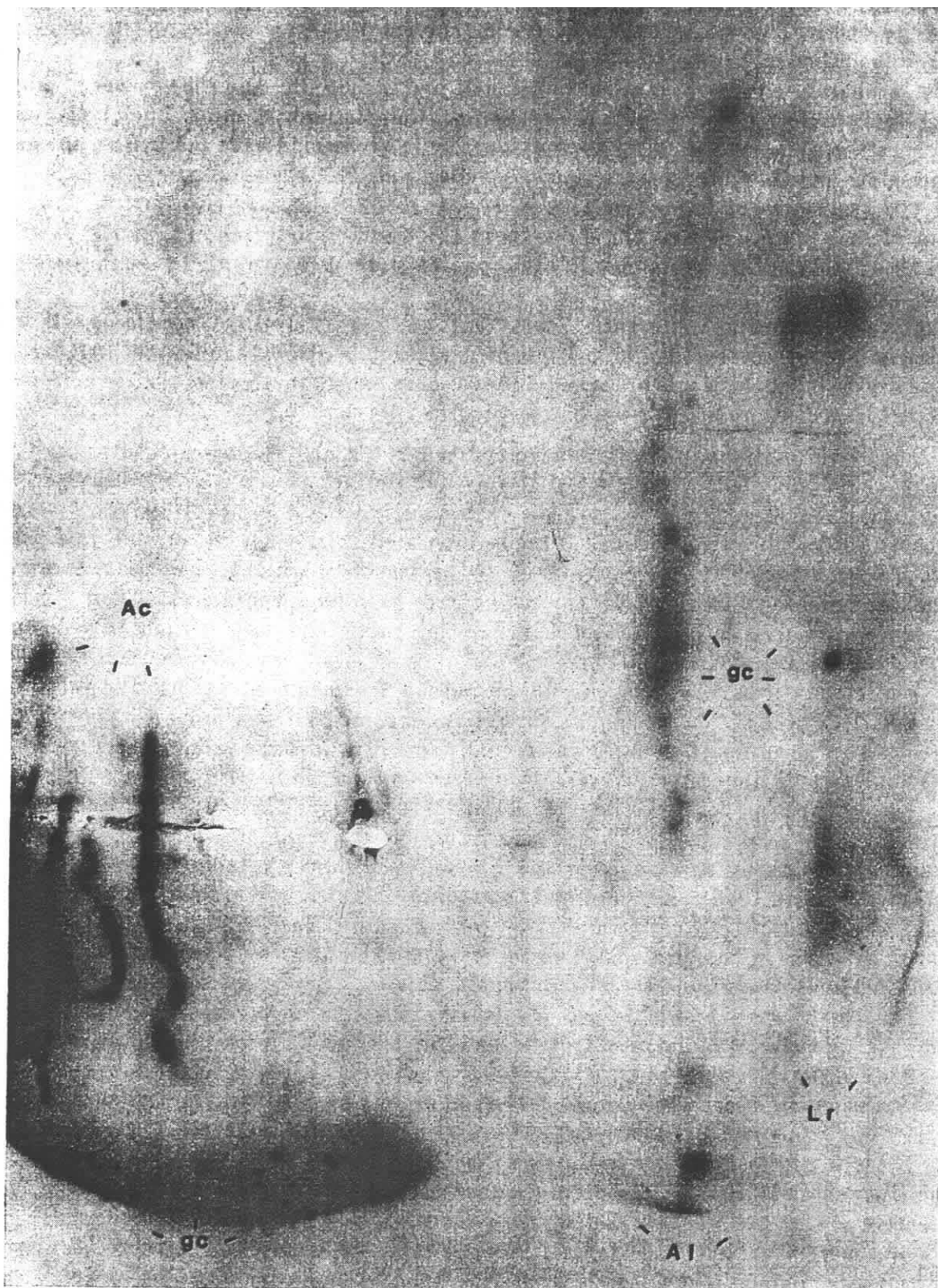


Fig. 2. Autoradiograph of earthworms taken from soil around ^{32}P labelled clover roots. Ac — *Aporrectodea caliginosa*; Al — *A. longa*; Lr — *Lumbricus rubellus*; gc — gut contents.

4. Discussion

The survey showed that clover roots labelled with foliarly applied ^{32}P could be used in the field to detect root-feeding animals.

Although the technique revealed the proportion of animals caught that had probably eaten some radioactive roots, the actual quantities of root ingested could not be determined by this essentially qualitative method.

Most of the dipteran larvae caught were *Bibio marci* larvae which is a polyphagous pasture pest; only one leatherjacket was found during the survey. None of the root-feeding fauna occurred in sufficient numbers to be serious clover pests.

The comparative rarity of labelled Collembola and nematodes, and the absence of labelled mites, highlight one problem with the technique. Calculations of mite, Collembola and nematode gut sizes showed that all of the soil fauna extracted could have marked an autoradiographic plate had they been feeding on radioactive root, but the small size of their gut and hence the small area of film exposed would have made them easy to overlook. It can be concluded that groups such as the mites lie at the limits of detectability, and so failure to reveal the presence of tracer on an autoradiograph is not conclusive proof that they were not feeding on *T. repens*.

The most interesting observation was that the earthworms: *Aporrectodea caliginosa*, *A. longa* and *Lumbricus rubellus* were frequently found to be strongly radio-labelled (Figure 2) and the degree of labelling was in excess of that seen in known rootfeeders such as coleopteran larvae. There are a number of possible explanations for this:

1. The earthworms had become contaminated through the leaking of ^{32}P from the roots into the surrounding soil which they then ingested during the six hour labelling period or during heat extraction, for those animals that were not hand-sorted. However, the initial laboratory work showed no evidence of the isotope leaking from the clover roots into the soil during a 6 hour period. Furthermore none of the known non-root feeding animals, such as spiders and ants, were labelled, as they might have been from contaminated soil. Also heat extracted earthworms left in contact with labelled roots for longer periods than hand extracted ones did not pick up more tracer.

2. The earthworms had accidentally picked up fragments of radioactive root whilst burrowing. However, if this was so, one might have expected a higher proportion of tunnelling earthworms to be radioactive than was actually found, whilst the radio active worms contained considerable amounts of tracer.

3. The earthworms had transintegumentarily absorbed ^{32}P directly from the living root by prolonged physical contact. This possibility was not investigated, although transintegumentary absorption by *Eisenia fetida* and *L. rubellus* has been established for amino acids (RICHARDS & ARME 1979), carbohydrates (RICHARDS & ARME 1980a) and for sodium acetate (RICHARDS & ARME 1980b). However, this work has all been conducted *in vitro*, with the worms in solutions of the particular substances, and there is no evidence that materials can be absorbed in the field from intact plant roots.

4. The earthworms had obtained ^{32}P from consuming mycorrhizal symbionts of the clover root. Transfer of radiotracer from roots to mycorrhizae has been demonstrated (READ *et al.* 1985) although in this study, no labelled hyphae were ever seen on the autoradiographs. Also, once the main roots were removed, the soil around labelled roots remained uncontaminated.

5. The earthworms had been feeding on the labelled clover roots. There have been reports in the literature (McRILL 1974, PEARCE 1978) that some earthworms eat roots and the implication of the present study is that *Lumbricus rubellus*, *Aporrectodea caliginosa*, *A. longa* and a number of enchytraeids fed on labelled roots, whereas *Allolobophora chlorotica*, *A. rosea* and two unidentified annelids did not, although the latter numbers are very small. The evidence for some form of root-feeding seems particularly strong for the hand-sorted earthworms. Indeed, together with the coleopteran larvae, they were the most frequently labelled group.

A possible objection to direct feeding on living roots being the route of tracer uptake is that as the extracted worms could continue feeding for many hours after the hand-sorted worms had been removed, more radioactive annelids should have been extracted by heat rather than by hand. However the lengthy time needed for heat extraction might permit much of the ingested radioactive material to be defecated, whilst the heating and drying might inhibit feeding. *A. caliginosa* can pass ingested food in as little as five hours when

introduced to new soil (BARLEY 1959); *A. rosea* has a gut turnover rate of 1–2.5 hours (BOLTON & PHILLIPSON 1976), and *L. rubellus* takes one day to pass ingested soil. However, the fact that some heat extracted earthworms were radioactive 7 days after labelling suggested that some ^{32}P had been assimilated.

These observations that earthworms, not normally regarded as consumers of living roots, were the greatest ingesters of ^{32}P from clover roots in this pasture implies that more work needs to be done on the identify^{in a} of root feeding invertebrates before calculations about the extent of root herbivory (SCOTT *et al.* 1979) can be regarded as having any precision.

5. Acknowledgements

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6. Zusammenfassung

(Eine radiometrische Prüfung von Wirbellosen, die lebende Kleewurzeln (*Trifolium repens*) verzehren, mit Hilfe von ^{32}P)

Kleewurzeln wurden im Freiland mit ^{32}P markiert, und die indirekt markierte Bodenfauna wurde autoradiographisch ermittelt. In der Reihenfolge ihrer Bedeutung waren es folgende Tiergruppen, die markierte Kleewurzeln verzehrten: Regenwürmer (*Aporrectodea longa*, *A. caliginosa* und *Lumbricus rubellus*), Rüsselkäferlarven, Dipterenlarven (*Bibio marci*) und einige Collembola (Entomobryidae).

Schlüsselwörter: Rhizophage, Wirbellose, ^{32}P , Radiotracer, *Trifolium repens*, Weide, Regenwürmer.

7. Literature

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Synopsis: *Original scientific paper*

BAYLIS, J. F., J. M. CHERRETT & J. B. FORD, 1986. A survey of the invertebrates feeding on living clover roots (*Trifolium repens* L.) using ^{32}P as a radiotracer. *Pedobiologia* **29**, 201—208.

Clover roots were labelled in the field with ^{32}P and the radioactive soil fauna were detected by autoradiography. The animals which consumed labelled clover roots were, in order of importance, earthworms (*Aporrectodea longa*, *A. caliginosa* and *Lumbricus rubellus*), weevil larvae, dipteran larvae (*Bibio marci*) and a few Collembola (family: Entomobryidae).

Key words: Root-feeding, invertebrates, ^{32}P , radiotracer, *Trifolium repens*, pasture, earthworms.